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Osmotic Repression of Anaerobic Metabolic Systems in *Escherichia coli*

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The influence of the osmolarity of the growth medium on anaerobic fermentation and nitrate respiratory pathways was analyzed. The levels of several enzymes, including formate dehydrogenase, hydrogenase, and nitrate reductase, plus a nickel uptake system were examined, as was the expression of the corresponding structural and regulatory genes. While some functions appear to be only moderately affected by an increase in osmolarity, others were found to vary considerably. An increase in the osmolarity of the medium inhibits both fermentation and anaerobic respiratory pathways, though in a more dramatic fashion for the former. *fnr* expression is affected by osmolarity, but the repression of anaerobic gene expression was shown to be independent of FNR regulatory protein, at least for *hyd-17* and *fdhF*. This repression could be mediated by the intracellular concentration of potassium and is reversed by glycine betaine.

Living organisms have evolved a variety of adaptive mechanisms to cope with changes in the osmotic strength of their environment (53). Bacteria require the presence of an outwardly directed turgor pressure to grow. When the osmotic pressure of the growth medium is increased, the maintenance of a relatively constant turgor pressure is initially achieved through an increase in the cytoplasmic potassium concentration (11, 12, 15). The ability of cells to grow under conditions of elevated osmolarity is determined both by the salt tolerance of their enzymes and by their capacity to accumulate compatible solutes in place of salts (12, 21). Some compatible solutes can alleviate the inhibitory effects of high osmolarity when they are present in the culture medium, suggesting that they can accumulate to high concentrations by transport but not by de novo synthesis. Compounds that have this ability are called osmoprotectants. Two mechanisms have been proposed for their modes of action: establishment of intracellular osmolarity and stabilization of biological macromolecules. In members of the family *Enterobacteriaceae*, glycine betaine was observed to be the most preferable osmoprotectant among all those studied (27). The majority of studies concerning osmoprotection by glycine betaine in *Escherichia coli* have focused on its accumulation through the ProP and ProU transport systems (7, 8, 12). Therefore, the *proU*, *ompF*, and *ompC* genes and their products were subject to most of the investigations (11, 12). On the other hand, most of the osmoregulated genes isolated to date are osmo-inducible (16, 17). An exception was observed for *mdoA* and *mdoB* involved in the biosynthesis of periplasmic membrane-derived oligosaccharides, which are osmorepressible (25). However, these later studies were carried out under aerobic growth conditions.

Facultative anaerobes, such as *E. coli*, can grow under aerobic as well as anaerobic conditions. They derive energy by either respiration or fermentation, depending on the availability of oxygen, carbon source, and electron acceptors (28, 40). Given a selection of electron acceptors, *E. coli* will preferentially choose oxygen and then nitrate, before using other less energetic acceptors, such as nitrite, fumarate, dimethyl sulfoxide, or trimethylamine nitrogen oxide (28). In the absence of oxygen and in the presence of nitrate, *E. coli* synthesizes the anaerobic formate-nitrate reductase respiratory chain. It consists of a formate dehydrogenase (FDH-N) and a nitrate reductase (NAR). It can generate a proton motive force by concomitant transfer of electrons from formate to nitrate coupled to proton translocation (46). Expression of structural genes for FDH-N and NAR enzymes is under the control of the general anaerobic regulatory protein FNR (4). In the absence of an exogenous electron acceptor, anaerobic acidification of the medium results in the biosynthesis of the formate-hydrogen lyase system that converts formate to carbon dioxide and molecular hydrogen. This multienzyme complex includes a second formate dehydrogenase (FDH-H), one or two unidentified electron carriers, and hydrogenase (HYD) isoenzyme 3 (44). Synthesis of these enzymes is repressed by oxygen and anaerobically by nitrate. It is induced in the presence of formate (46). The anaerobic FNR regulator is not required for expression of the *fdhF* structural gene for FDH-H (49), but it regulates HYD activity through its effect on the *nik* operon which encodes the specific nickel transport system (50, 51).

Most physiological approaches for osmotic stress and osmoprotectant accumulation were tested with cells grown aerobically. In *Salmonella typhimurium*, an overlap between osmotic and anaerobic stress responses was shown (32); osmotically induced expression of *proU* requires anaerobic growth for optimum induction; this effect was not observed in *E. coli* (43). Nevertheless, Ni Bhriain et al. (32) suggest that many anaerobic pathways are regulated by overlapping regulations mediated by osmotic adaptation. In this communication, we report the influence of medium osmolarity on

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TABLE 1. Bacterial strains

Strain	Genotype	Reference or source
MC4100	*araD139* Δ(*lac*)U169 *thi rpsL*	9
LCB79	MC4100 *narG-lac*	36
LCB311	MC4100 *fnr-lac*	35
HYD723	MC4100 *nik-lac*	46
FD71	MC4100 *fdhF-lac*	49
VJS1224	MC4100 *fdnG-lac*	4
M17s	MC4100 *hyd-17–lac*	37
M17sF	M17s *nirR22* (*fnr*) *zjc-261*::Tn*10*	L. MacWalter
FD71F	FD71 *nirR22* (*fnr*) *zjc-261*::Tn*10*	This laboratory

anaerobic enzyme activity and gene expression, using the formate-hydrogen lyase and formate-nitrate reductase pathways as model systems.

MATERIALS AND METHODS

Bacterial strains and culture media. All the bacterial strains used are derivatives of *E. coli* K-12 strain MC4100 and are listed in Table 1.

Organisms were grown aerobically or anaerobically on either LB or M63 glucose medium (30) supplemented with 2 μM sodium selenite and 2 μM ammonium molybdate. When required, LB and M63 media were supplemented with either sodium formate at concentrations of 30 mM and 1 mM, respectively, or potassium nitrate at 100 mM or 10 mM, respectively. Anaerobic growth was achieved in 250-ml bottles filled almost to the top, inoculated with 20 ml of overnight cultures grown in the same medium, and tightly stoppered to maintain anaerobiosis. Incubations were carried out at 37°C. For anaerobic enzyme activity assays, cells were harvested in the mid-exponential phase of growth.

Expression of operon fusions was measured by monitoring β-galactosidase activity in overnight-grown cells, the activity during the exponential phase showing the same evolution. For the *fdhF*, *hyd-17*, and *nik* genes, formate was added as an inducer, whereas the *narG* and *fdnG* genes were tested in the presence of nitrate.

Alternatively, for growth measurements, cultures were grown in a fermentor (Biolafitte) filled with 1 liter of medium, bubbled with nitrogen (50 liters/h), and continuously stirred (60 rpm).

The osmolarity of the growth medium was increased by adding various concentrations of NaCl up to 0.7 M or other osmolytes and determined by measuring the freezing point with an osmometer.

Enzyme assays. Cells were washed twice and harvested in phosphate Na^+ and K^+ buffer (pH 7, 100 mM). FDH-H, FDH-N, HYD, NAR, and β-galactosidase were measured spectrophotometrically as described earlier (48). The enzymatic activities are expressed as micromoles of formate oxidized per minute per milligram of proteins for FDH-H and FDH-N, as micromoles of benzyl viologen reduced per minute permilligram of proteins for HYD, and as micromoles of nitrate reduced per minute per milligram of proteins for NAR; the values are the means for at least five independent experiments, and in each case, the standard deviation was less than 10%. The relationship of an *E. coli* cell suspension at an optical density at 570 nm and the corresponding amount of protein was determined and used for the determination of specific activities.

Potassium assay. Cells were grown to the mid-exponential phase in M63 medium containing NaCl and glycine betaine as appropriate. Cultures were harvested by centrifugation (10,000 × *g*, 5 min) and washed with an isotonic solution of sorbitol. After centrifugation (10,000 × *g*, 5 min), the pellets were resuspended in 0.1 N HCl and incubated overnight at room temperature. The suspension was centrifuged, and the K^+ concentration was determined in the supernatant by flame photometry (PHF80; Isabiology).

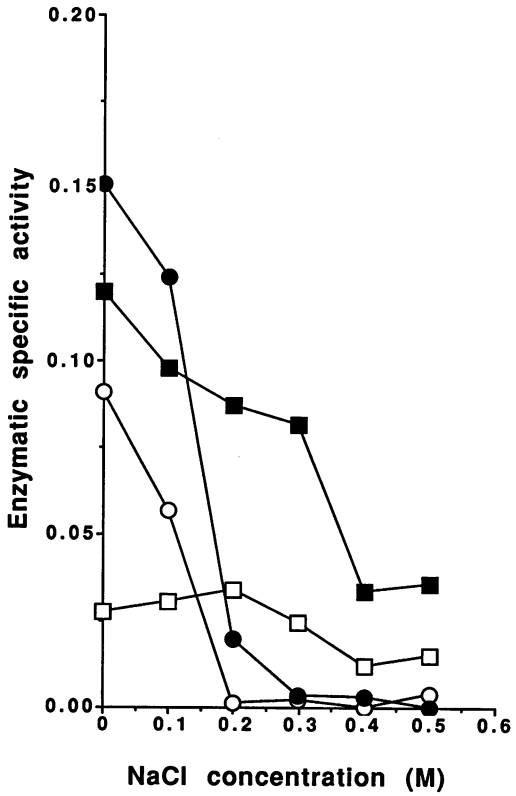

FIG. 1. Influence of medium osmolarity on FDH-H, FDH-N, HYD, and NAR activities. Cells were grown anaerobically in M63 glucose medium containing 1 mM formate, 2 μM selenite, 2 μM molybdate, and increasing concentrations of NaCl. Enzymatic activity, as described in Materials and Methods, was assayed in the mid-exponential phase of growth. Specific activities for FDH-H (○), HYD (●), FDH-N (□), and NAR (■) are shown.

RESULTS

Influence of osmotic stress on anaerobic formate metabolism enzymes. The influence of osmolarity on two well-established anaerobic metabolic pathways was analyzed. The activities of the two components of the formate-hydrogen lyase system, FDH-H and HYD, decreased rapidly with the increase of osmolarity in the growth medium (Fig. 1). These two enzymes were extremely susceptible to the medium's osmolarity; their activities were completely abolished at 0.2 M and 0.3 M NaCl for FDH-H and HYD, respectively. On the contrary, FDH-N and NAR were rather salt tolerant, although anaerobic growth in the presence of nitrate was inhibited by NaCl at a level comparable to that of fermentative growth. FDH-N activity remained constant up to 0.3 M NaCl but was reduced to 55% of the initial level with 0.5 M NaCl. NAR activity reached 70% of the initial level with 0.3

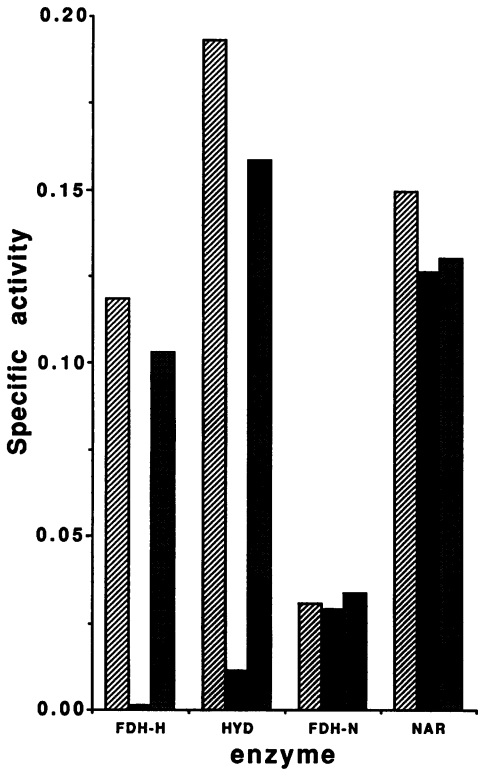

FIG. 2. Restoration of enzymatic activity by glycine betaine. Cells were grown anaerobically to the mid-exponential phase in M63 glucose medium supplemented with 1 mM formate, 2 μM selenite, and 2 μM molybdate and containing no added osmolyte (▨), 0.3 M NaCl (■), or 0.3 M NaCl and 1 mM glycine betaine (▩).

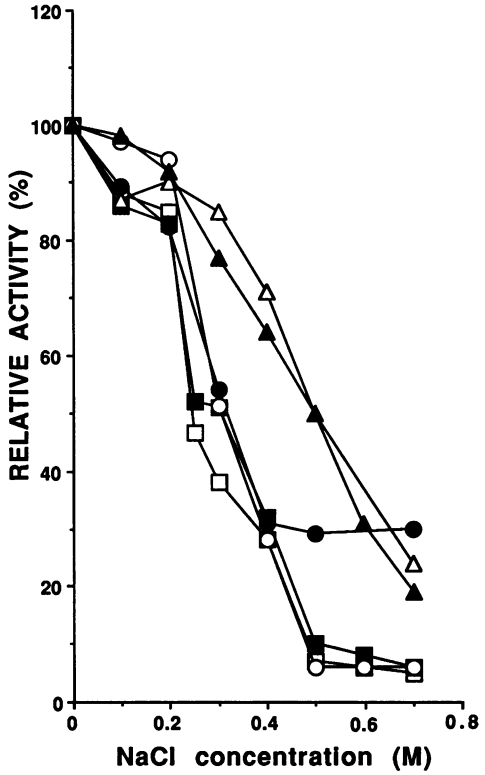

FIG. 3. Influence of increasing medium osmolarity on expression of anaerobic genes. Cells were grown anaerobically in M63 glucose medium containing 1 mM formate, 2 μM selenite, and 2 μM molybdate. NaCl was added at the indicated concentrations. After 10 generations in the same medium, cells were collected in the mid-exponential-growth phase, and β-galactosidase activity was assayed. Results are averages for at least three different experiments and expressed as the percentage of the activity obtained in M63 medium deprived of NaCl. The 100% values are identical to those described in Table 2. Symbols: ▲, VJS1224 (*fdnG-lac*); △, LCB79 (*narG-lac*); ■, HYD723 (*nik-lac*); □, FD71 (*fdhF-lac*); ●, LCB311 (*fnr-lac*); ○, M17s (*hyd-17-lac*).

M NaCl and was then reduced to 30% at higher NaCl concentrations (Fig. 1).

In order to analyze its osmoprotective effect on the enzymes involved in the anaerobic formate degradation, 1 mM glycine betaine was added to the growth medium containing 0.3 M NaCl. As expected, glycine betaine did not have any significant influence on the FDH-N and NAR activities (Fig. 2). In contrast, it allowed a full recovery of FDH-H and HYD activities (Fig. 2). Thus, in a medium containing 0.3 M NaCl, 1 mM glycine betaine was able to completely reverse osmotic inhibition of the formate-hydrogen lyase complex.

Osmotic regulation of anaerobically expressed structural or regulatory genes. The selenocysteine-containing enzyme FDH-H is encoded by the *fdhF* structural gene located at 93 min on the chromosome of *E. coli* (49, 54). As reported above (Fig. 1), the activity of FDH-H was totally abolished in medium with low osmolarity (0.2 M NaCl). To test the possibility of an effect on the transcriptional regulation of the *fdhF* expression, we monitored the expression of the *fdhF-lacZ* operon fusion of strain FD71 in medium with increasing osmolarity. The β-galactosidase activity decreased to approximately 40% of the initial level in the presence of 0.3 M NaCl in the growth medium. At 0.5 M NaCl, it was further reduced to less than 10% of the initial level (Fig. 3). This result is in good accordance with the repression of FDH-H activity by osmotic stress reported above (Fig. 1), although the response of gene expression to increasing salt concentrations showed a slight delay compared with that of enzyme activity.

Under anaerobic conditions, *E. coli* synthesizes some or all of the three HYD isoenzymes, depending on the growth medium (5, 44). HYD 2 catalyzes the oxidation of hydrogen, while HYD 3 and 1 are responsible for production and recycling of hydrogen during fermentative growth, respectively. A fusion mutant (strain M17s), specifically devoid of HYD 3 activity (6), was used to monitor the behavior of a structural operon encoding the components of the fermentative HYD associated with the formate-hydrogen lyase pathway. Since expression of the *hyd-17-lacZ* fusion is affected by osmolarity, the results are similar to those obtained for the *fdhF-lacZ* fusion (Fig. 3). In the presence of 0.3 M NaCl, the β-galactosidase activity was reduced approximately 50%, and it was lower than 10% of the initial level for NaCl concentrations greater than 0.5 M.

As described previously, the *nik* operon is involved in specific nickel transport and is required for the expression of all three HYD isoenzymes (50). β-Galactosidase activity expressed from the *nik-lacZ* fusion decreased progressively when increasing amounts of NaCl were added to the growth medium (Fig. 3). It was reduced to less than 50% of the initial activity in the presence of 0.3 M NaCl, consistent with the

TABLE 2. Effect of various osmolytes and glycine betaine on the expression of *lac* fusions[a]

Medium additive	β-Galactosidase relative activity in strain:					
	LCB311 (*fnr-lac*)	HYD723 (*nik-lac*)	FD71 (*fdhF-lac*)	M17s (*hyd-17–lac*)	VJS1224 (*fdnG-lac*)	LCB79 (*narG-lac*)
None	100 (3,100)	100 (110)	100 (230)	100 (300)	100 (400)	100 (220)
NaCl	54	51	38	51	77	85
NaCl + 1 mM glycine betaine	72	94	82	96	99	101
KCl	54	49	18	43	79	122
Mannitol	50	32	7	43	86	79
Glycerol	92	94	80	101	98	110
Sucrose	66	50	36	78	121	96

[a] All cultures were grown anaerobically to the mid-exponential phase in M63 medium with low or high osmolarity. High osmolarity was 820 mosM, corresponding to 0.3 M NaCl. It was created by the addition of various osmolytes, electrolytes, or nonelectrolytes and the permeant molecule glycerol. Cells were grown in the same medium for six generations before β-galactosidase activity was assayed. Results are the averages for at least three independent experiments and expressed as a percentage of specific enzymatic activity measured in medium deprived of NaCl. β-Galactosidase specific activity (nmol min^{-1} mg of $proteins^{-1}$) of cells grown without NaCl is indicated in parentheses for each strain.

alteration of HYD activity by osmotic stress described above (Fig. 2).

The second formate dehydrogenase in *E. coli* is FDH-N, encoded by the *fdnGHI* operon. This enzyme is associated with the major respiratory NAR to form an enzymatic complex induced by nitrate during anaerobic growth. We used an *fdnG-lacZ* fusion in an attempt to analyze the osmotic regulation of the *fdnGHI* operon expression. Consistent with its effect on the FDH-N activity (Fig. 2), the presence of 0.3 M NaCl had a marginal effect on the expression of *fdnGHI* (less than 30% inhibition). As the NaCl concentration increased, β-galactosidase activity decreased to 20% of the initial level at 0.7 M NaCl.

The *narGHJI* operon (formerly called *chlC*) encodes the major anaerobic NAR of *E. coli* (46). At a concentration of 0.3 M, NaCl barely had an effect on *narGHJI* expression, while at higher concentrations, 0.5 M and 0.7 M, it repressed β-galactosidase activity to approximately 50 and 24% of the initial level, respectively (Fig. 3). This observation is also in good agreement with the previously reported osmotic effect of NaCl on NAR activity (Fig. 1).

The *fnr* gene product is required for the anaerobic expression of several enzymes including those of the formate degradative pathway, namely FDH-H, HYD, FDH-N, and NAR (45, 48, 49). Thus, we wished to examine the possible effects of salts on *fnr* expression. The β-galactosidase activity expressed by an *fnr-lacZ* mutant strain decreased progressively with the increase of the NaCl concentration in the growth medium (Fig. 3). It reached 30% of the initial level at 0.4 M NaCl and then became insensitive to a further increase in the NaCl concentration. Since *fnr* expression is influenced by the osmolarity of the medium, the anaerobic repression of gene expression by osmolarity could result from the decrease of FNR concentration. Most of the genes analyzed are directly controlled by FNR and are not expressed in an *fnr* strain; only *fdhF* and *hyd-17* are expressed in an *fnr* mutant in medium containing formate. To test the role of FNR in osmotic repression, we analyzed the expression of *hyd-17–lac* and *fdhF-lac* fusions in strains M17s (*hyd-17–lac*), M17sF (*hyd-17–lac fnr*), FD71 (*fdhF-lac*), and FD71F (*fdhF-lac fnr*). When 0.3 or 0.6 M NaCl was added to the medium, the drop in β-galactosidase activity was similar in the presence or absence of *fnr* background (results presented in Fig. 3 for M17s and FD71). Therefore, FNR is not directly involved in osmotic repression of anaerobic gene expression; nevertheless, the decrease in the FNR concentration could result in part in the observed repression of *fdnGHI*, *narGHJI*, and *nik* expression.

Inhibitory and repressive effects of NaCl on the anaerobic enzymes and the corresponding genes might result from the high concentration of Na^+ or Cl^- ions in the growth medium rather than from a high osmolarity. To test this hypothesis, the effect of other osmolytes, such as KCl, sucrose, or mannitol, was examined (Table 2). These compounds were used at concentrations generating the same osmotic strength as that with 0.3 M NaCl (820 mosM). All of them displayed repressive effects on the various fusions tested similar to those described above with NaCl, whereas the diffusible molecule glycerol had no effect. Thus, inhibition of gene expression resulted from osmotic stress rather than from salt stress. Furthermore, addition of the osmoprotectant glycine betaine in the growth medium led to the restoration of expression of the fusions in the presence of NaCl (Table 2). Thus, the repressive effect of NaCl and other osmolytes was a consequence of fluctuations in the osmolarity of the growth medium.

In vitro effect of NaCl on formate metabolism enzymes. When present at 0.3 M in the growth medium, NaCl had more effect on FDH-H activity (100% inhibition, Fig. 1 and 2) than on the *fdhF* structural gene expression (60% repression, Fig. 3). This observation was also true for the HYD activity and the expression of *hyd-17* and of the *nik* locus. It suggests that posttranscriptional and (or) posttranslational effects might exist. The effect of osmolarity on FDH-H, HYD, FDH-N, and NAR was analyzed in vitro by incubation of crude extracts with 0.3 M NaCl prior to enzyme assay. As shown in Fig. 4, only FDH-H activity was significantly altered. Other activities were unaffected or only slightly enhanced. *E. coli* cells submitted to osmotic stress accumulate glycine betaine, when it is present in the growth medium. The intracellular concentration of glycine betaine can reach values of 0.5 to 0.7 M (26, 38). The addition of 0.5 M glycine betaine to the incubation medium containing 0.3 M NaCl resulted in only a slight stimulation of FDH-H, whether the glycine betaine was added 30 min before or at the same time as NaCl. Furthermore, this recovery was very weak compared with the full protection of this enzyme in vivo (60% against 100%).

Intracellular potassium content under high-osmolarity growth conditions. The primary response of bacteria to the increase of osmolarity in the medium is the intracellular accumulation of potassium ions. When osmoprotectants are

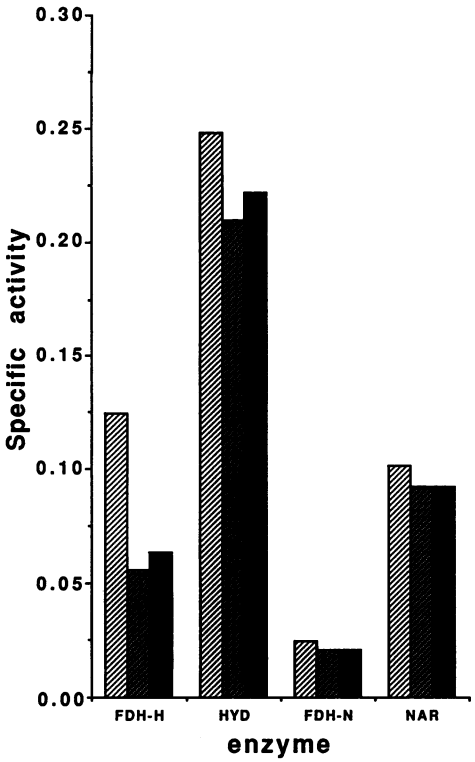

FIG. 4. Effect of NaCl and glycine betaine on enzymatic activities in crude extracts. Strain MC4100 was grown anaerobically in M63 glucose medium containing 1 mM formate, 2 μM selenite, and 2 μM molybdate in the absence of NaCl. Cells were collected by centrifugation and resuspended in 50 mM phosphate buffer (pH 6.8). They were permeabilized with toluene (50 μl · ml of cell suspension^{-1}). Enzymatic activities were assayed with cells incubated in the same buffer (▨), in the buffer supplemented with 0.3 M NaCl (■), or in the buffer supplemented with 0.3 M NaCl and 0.5 M glycine betaine for 30 min at room temperature (■).

present in the growth medium, they are accumulated in the cells in a second phase, which results in the excretion of K^+ ions. In most eubacteria, glycine betaine is the preferred compatible solute. We have determined the K^+ content in MC4100 and its derivatives grown in M63 medium in the presence or absence of 0.3 M NaCl and 1 mM glycine betaine. The results obtained for the derivatives were identical to those for the parental strain MC4100 presented in Table 3 and similar to those found by Sutherland et al. (47) for MC4100 aerobically grown cells in media of identical osmolarity. Whatever the growth conditions used, fermentation or anaerobic respiration, 0.3 M NaCl enhanced the intracellular potassium concentration. In all cases, the K^+ level decreased in the presence of 1 mM glycine betaine, although the potassium concentration was still 25% higher than that obtained in the absence of NaCl.

Growth inhibition by salt stress. While nitrate respiration pathway gene expression and enzymes appear to be only moderately affected by an increase in osmolarity, fermentation pathway gene expression and enzymes are extremely sensitive to an increase in the medium's osmolarity. Therefore, we tested the influence of the elevation of osmotic pressure of the medium on aerobic and anaerobic respiration and fermentation growth of wild-type strain MC4100 (Fig. 5). The different growth conditions (aerobic, anaerobic in the presence of nitrate, and anaerobic in the absence of nitrate) showed the same effect on growth parameters. Generation time and growth yield were reduced approximately 50 and 35%, respectively, with 0.3 M NaCl in M63 minimal growth medium. Further, the inhibition was fully abolished by the addition of 1 mM glycine betaine to the culture medium. However, the inhibition of growth by NaCl was not observed when cells were grown in LB medium (data not shown). It might result from the natural presence of glycine betaine in yeast extract as reported by Dulaney et al. (14).

DISCUSSION

In this paper, we report a general repression phenomenon caused by osmotic stress on the anaerobic formate metabolism. This regulation affects both enzyme activity and gene expression.

The regulation of the expression of osmoresponsive genes is actually explained by two mechanisms. (i) Increase in the osmolarity of the growth medium leads to an increase in supercoiling of DNA, which in turn affects gene expression (18–20); (ii) growth in high-osmolarity medium is associated with intracellular accumulation of potassium glutamate, which was shown to directly control *proU* expression (24, 41, 42). These two mechanisms might be complementary to each other rather than exclusive.

Aside from its response to osmotic stress, DNA supercoiling was also involved in the regulation of anaerobic gene expression (13, 32, 33, 52). The relationship between gyrase-dependent and FNR-dependent anaerobic regulation has been observed by investigators in different laboratories. Among the gyrase-requiring genes, those encoding dimethyl sulfoxide reductase, uptake HYD, and FDH-N are under the control of the FNR protein (3, 23). Conversely, the expression of the FNR-independent *fdhF* gene, which encodes FDH-H, was enhanced by agents that inhibit DNA gyrase activity (3). However, no correlation between osmotic repression and FNR regulation was observed in our studies (Fig. 3). Firstly, considering the expression of the three FNR-dependent genes examined, *nik* has a greater sensitivity to the increase of osmolarity than *fdnG* and *narG*. Secondly, the FNR-independent gene *fdhF* shows the same response to osmotic stress as does *nik*. Thirdly, *fnr* follows the same pattern of repression as *nik* and *fdhF* at concentrations of NaCl below 0.4 M. Fourthly, *fnr* does not have any effect on osmotic repression of *hyd-17* and *fdhF* gene expression. However, when the osmolarity of the growth medium

TABLE 3. Potassium content of cells grown anaerobically in conditions of osmotic constraint[a]

Medium additive	Potassium content (mM) with growth as:	
	Fermentation	Anaerobic nitrate respiration
None	915	840
0.3 M NaCl	1,525	1,440
0.3 M NaCl + 1 mM glycine betaine	1,135	1,023

[a] Cells of strain MC4100 were grown in M63 containing the appropriate additive(s) as indicated. Fermentative growth was achieved by adding 0.4% glucose and 1 mM formate to M63, whereas anaerobic respiration-dependent growth was carried out in M63 containing 0.4% glucose and 10 mM $NaNO_3$ (or KNO_3). Cells were grown for 10 generations in the same medium and harvested in the mid-exponential phase for potassium assay. Data are the averages for at least three independent experiments.

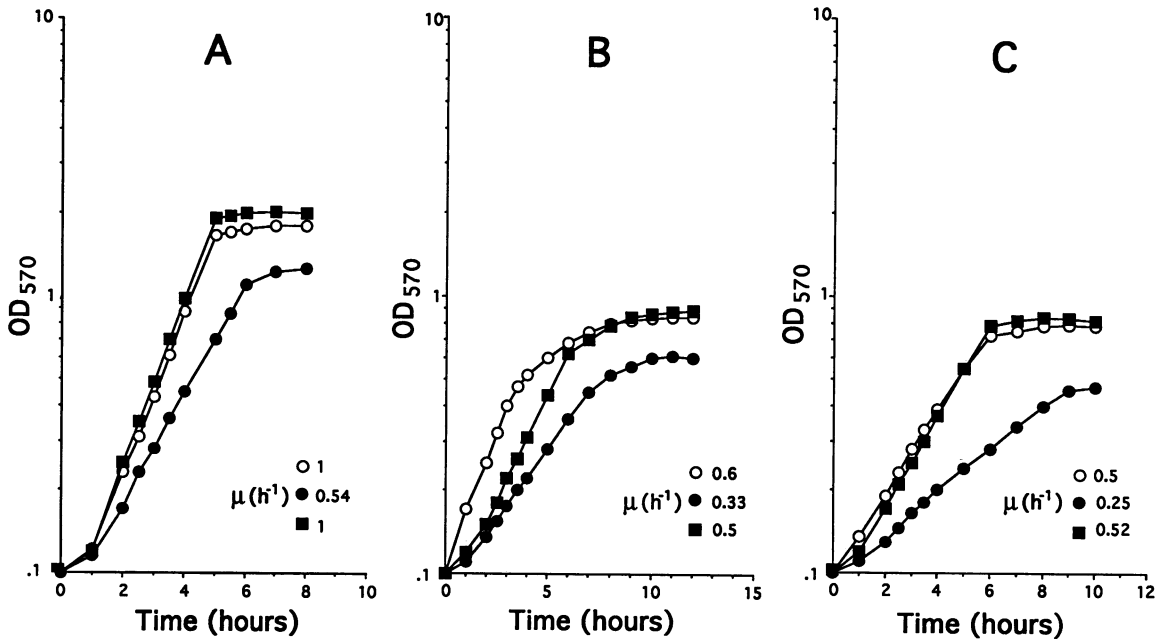

FIG. 5. Influence of medium osmolarity on growth of strain MC4100. Strain MC4100 was grown in M63 glucose medium containing 1 mM formate, 2 μM selenite, and 2 μM molybdate supplemented with NaCl and glycine betaine as appropriate. Cultures were grown under aerobic conditions (A), anaerobic conditions in the presence of nitrate (B), or anaerobic conditions in the absence of nitrate (C). Symbols: ○, no added osmolyte; ●, 0.3 M NaCl; ■, 0.3 M NaCl and 1 mM glycine betaine. Generation times (μ) are indicated for each curve. OD_{570}, optical density at 570 nm.

was increased, expression of *fnr* stayed at a level comparable to that of *narG*, which was consistently higher than that of *nik* and *fdhF*. These results are consistent with an FNR-unrelated osmotic regulatory system.

Indeed, according to the second mechanism, we found that intracellular K^+ levels were increased twofold in response to the addition of NaCl to the growth medium. Since this accumulation can be reversed by glycine betaine, the phenomenon must reflect an osmoprotection. Both inhibition of enzymatic activities and repression of gene expression are in good accordance with the intracellular concentration of potassium. As a consequence, the osmotic repression of anaerobic formate metabolism might be mediated by the control of the potassium concentration.

Osmotic stress not only affects the expression of anaerobic genes but also could directly influence the activity of FDH-H. This action was shown in vitro in crude extracts, and it could contribute in vivo to the reduction of FDH-H activity. We noted that enzymatic activity was reduced to a greater extent than the inhibition of *fdhF* expression. In vitro FDH-H is the only enzymatic activity of these pathways which is reduced in the presence of NaCl.

Elevated concentrations of salts typically increase K_m values to levels at which the regulatory responsiveness of enzymes becomes suboptimal (53). Some effects of inorganic ions result from their binding to active sites or from an alteration of protein stability and compartmentalization in the cell structures. Osmolyte protection of enzymes has received much attention (53); various organic compounds, such as betaines, used as osmolytes by organisms, can enhance the stability of proteins and membranes in environments of low water activity (1, 10, 22, 31, 34, 39). Since the response of anaerobic gene expression to the increase of osmolarity shows a delay compared with that of enzymatic activities (Fig. 1 and 3), posttranscriptional and posttranslational modifications should exist. Anaerobic formate metabolism is mediated via multisubunit enzymes requiring the incorporation of metals, such as Fe, Mo, Se, and Ni, for activity (46). Therefore, these modifications could happen during the subunit assembly, during metal incorporation into these metalloenzymes, or during their translocation into the membrane. The in vitro assay demonstrated that only FDH-H was significantly inhibited by NaCl (Fig. 4), and the same inhibition was reported for the purified enzyme (2). Furthermore, this inhibition could not be reversed by glycine betaine. Moreover, restoration or osmoprotection of the enzymes was shown to depend on protein biosynthesis, because addition of glycine betaine to the growth medium completely reverses the activities (Fig. 2). Therefore, further studies are necessary to elucidate the mechanism of the posttranscriptional modifications.

The formate-hydrogen lyase pathway was more sensitive to osmotic stress than the formate-nitrate respiratory chain. However, anaerobic growth was inhibited identically under conditions of both fermentation and formate-nitrate respiration. This fact suggests that bioenergetic pathways are not limiting steps for growth under osmotic stress; one or various steps in the metabolism must be affected, one putative goal being DNA replication (29).

Most osmoregulated genes isolated today are osmoinducible (16, 17). Only *mdoA* and *mdoB* were reported to be osmorepressible (25). All these genes were characterized in aerobically grown cells. By contrast, *fdhF*, *hyd-17*, and *nik* are expressed only under anaerobic growth conditions. Actually *fdhF*, *hyd-17*, and *nik* belong to a small family of osmorepressible genes. Screening of osmoresponsive genes belonging to those induced under various conditions would allow the identification of new candidates. Further study will

help in the characterization of the mechanisms of gene expression control by osmolarity.

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